Asian Herpetological Research 2010, 1(2): 118-122
DOI: 10.3724/SP.J.1245.2010.00118

Genotoxicity of the Pesticide Dichlorvos and Herbicide Butachlor on *Rana zhenhaiensis* Tadpoles

GENG Baorong[1, 2 *], LIN Ling[1], ZHANG Qiujin[1, 2] and ZHONG Bijin[1]

[1] *College of Life Sciences, Fujian Normal University, Fuzhou* 350108*, Fujian, China*
[2] *Higher Education Laboratory of Developmental Biology and Neurobiology, Fujian Normal University, Fuzhou* 350108*, Fujian, China*

Abstract Genotoxicity of dichlorvos and butachlor on erythrocytes of *Rana zhenhaiensis* tadpoles was investigated by the alkaline single-cell gel electrophoresis assay or comet assay. Tadpoles were treated for 24 h in the laboratory with different concentrations of the testing agents, 2.256, 4.512, 6.768, 9.024, 11.280 mg/L for dichlorvos and 0.292, 0.584, 0.876, 1.168, 1.460 mg/L for butachlor, to use the comet Assay to test for the significance of dosage responsiveness to an increase in DNA damage, as measured by the mean DNA tail length-to-width ratio. The concentrations of 4.512 mg/L dichlorvos and 0.876 mg/L butachlor resulted in highly significant increases in DNA damage of the tadpoles. There were linear correlations between the mean DNA tail length-to-width ratio and the concentrations of the two test substances. Our results showed that the two commonly used agricultural chemicals caused dose dependent DNA damage of amphibians, and that comet assay might be a useful tool for measuring DNA damage of tadpoles exposed in the field.

Keywords comet assay, dichlorvos, butachlor, DNA damage, pesticide, herbicide, *Rana zhenhaiensis*

1. Introduction

Global decline in amphibian populations has increasingly attracted the attention of scientists in the recent decades (Stuart *et al*., 2004; Beebee and Griffiths, 2005; Xie *et al*., 2007), with diverse speculations regarding the causes and mechanisms for this decline being proposed (Ankley *et al*., 1998; Davidson, 2004; Wang and Jia, 2009).

Chemical contamination as a consequence of pesticide application continues to be postulated as a contributing factor in this decline (Berrill *et al*., 1997; Mann and Bidwell, 2001). Indeed, amphibians may be at greater risk of the toxic effects of pesticides than other aquatic vertebrates due to their highly permeable skin and preferred breeding habitats which consist of shallow, lentic or ephemeral water bodies (Duellman and Trueb, 1994) where contaminants may accumulate without substantial dilution.

Dichlorvos (2, 2–dichlorvinyl dimethylphosphate, $C_4H_7Cl_2O_4P$) and butachlor (N-butoxymethyl-a-chloro-2-6 diethylacetanilide, $C_{17}H_{26}ClNO_2$) are the most commonly used pesticide and herbicide in China. Dichlorvos is an organophosphate pesticide, and it is now widely used to kill insect pests in farming, forestry, and the general environment (WHO, 1989; ATSDR, 1997). Butachlor, as an herbicide, is now widely used in Asia and South America and is applied either pre-emergence or early post-emergence to control a large variety of annual grasses and some broad-leaved weeds. More than 10,000 tons (10^7 kg) of each is produced annually in China, with production expected to increase in the future (Hu, 1998; Yao *et a*l., 2003). Because the two chemicals have been frequently applied, their potential negative impacts need to be carefully considered.

Rana zhenhaiensis (Anura: Ranidae) is widely distributed in southeastern China. It was chosen as the test animal for this study due to its presence in many disturbed agricultural areas, and its reproductive period is relatively long (Cai, 1979). The chance for the tadpoles of *R. zhenhaiensis* to contact dichlorvos and butachlor is very high.

* Corresponding author: Prof. GENG Baorong, from College of Life Sciences, Fujian Normal University, with his research mainly focusing on taxonomy, ecology and ecotoxicology of amphibians.
E-mail: brgeng@fjnu.edu.cn
Received: 7 June 2010 Accepted: 11 November 2010

In addition, a previous biomonitoring study found that the amount of DNA damage detected in the tadpoles from agricultural areas was significantly higher than that in those from nonagricultural areas (Ralph *et al*., 1996).

Genotoxicity is a capacity of an agent to cause damage to the genetic material either directly or indirectly. Genotoxicity testing may help to explain or predict potential damaging degree of a chemical on living organisms or population. To provide the scientific information for protecting amphibian populations, and evaluating and monitoring water pollution using amphibians as bioindicator, we evaluated the DNA damage of *R. zhenhaiensis* tadpoles caused by dichlorvos and butachlor by using the single cell gel electrophoresis (SCGE) or comet assay.

2. Materials and Methods

2.1 Chemicals Dichlorvos was provided by the Dachen Pesticides Factory (Shandong, China), and butachlor by the Qingfeng Pesticides Corporation (Hangzhou, China). The general reagents and chemicals used for the comet assay were purchased from BBI (Ontario, Canada) and Sigma (St. Louis, USA).

2.2 Animals A total of 6 fertilized egg masses of *R. zhenhaiensis* were collected from farm fields in Rixi Town, about 40 km northwest from Fuzhou in Fujian Province, China and grown into middle-stage tadpoles with total length of about 15.28±2.01 mm (Gosner-stage 35-36; Gosner, 1960) in laboratory.

2.3 Treatment The maximum test concentrations used in the assays were based on the 96-h LD_{50} concentration for dichlorvos (11.280 mg/L) and butachlor (1.460 mg/L) (Geng *et al*., 2005). From these concentrations, a series of four 20% dilutions were used to make low concentrations down to 2.256 mg/L and 0.292 mg/L (Table 1), respectively. A 24-h exposure to all the concentrations resulted in 100% tadpole survival.

A total of 216 tadpoles were divided into three parts, with each part consisting of 72 individuals. Treatments were replicated 3 times. Exposures of 72 tadpoles, there were 6 tadpoles per group including negative, positive controls and various treated groups. The negative, positive controls and treated groups were conducted in the dark in 2-L beakers containing 1500 ml of dechlorinated water, 3.125 mg/L methyl methanesulfonate (MMS) and 5 varying concentrations of dichlorvos and butachlor, respectively. After exposure for 24 h, the tadpoles were removed and blood samples were collected immediately.

2.4 Comet assay To collect erythrocytes, the tadpoles were decapitated individually and placed immediately in 1 mL of cold phosphate buffered saline (PBS, calcium- and magnesium- free) for 5 min. Cometassay was performed as described previously (Geng *et al*., 2005a). In brief, each roughened microscope slide was coated with 90 μL of 0.7 % NMA at 37°C, and then covered with a coverslip and transferred to a humidified box at 4°C for 20 min to allow the solidification of agarose. Subsequently, the erythrocytes (15 μL) were mixed with 0.7 % LMA (60 μL), and then pipetted onto fully frosted slides and covered with coverslips. The slides were stored in the dark at 4°C to allow complete polymerization of the agarose. After removing the coverslips, the slides were immersed into freshly made lysing solution (2.5 mol/L NaCl, 100 mmol/L Na_2EDTA, 10 mmol/L Tris, 1% Triton X-100, 10% DMSO, pH 10) and incubated at 4°C in the dark for 2 h. Sequentially, the slides were drained and placed in an alkaline electrophoresis buffer (1 mmol/L Na_2EDTA, 300 mmol/L NaOH, pH 13) for 30 min. For the electrophoresis, the power supply was set at 20 V

Table 1 Detection of DNA damage, measured by the mean DNA length-to-width ratios, in erythrocytes of *R. zhenhaiensis* tadpoles after a 24-h whole-body exposure to dechlorinated water, MMS and various concentrations of dichlorvos.

	Dose (mg/L)	Number of tested tadpoles	DNA length : width ratio ± *SE*[a]	P[b]	Range: SD ratio
Dichlorvos	11.280	6	2.119 ± 0.591	<0.001	3.881
	9.024	6	1.751 ± 0.473	<0.001	4.347
	6.768	6	1.657 ± 0.589	<0.001	3.739
	4.512	6	1.474 ± 0.433	<0.001	5.069
	2.256	6	1.080 ± 0.336	<0.05	4.563
Negative control		6	1.018 ± 0.348		4.129
Positive control (MMS 3.125 mg/L)		6	1.898 ± 0.694	<0.001	4.636

[a]: Ratios based on 100 cells/tadpole.

[b]: All comparisons are relative to the negative control. The same below.

(0.714 V/cm) and the current was adjusted to 200 mA by slowly changing the buffer level in the tray. The slides were electrophoresed in the dark at 4°C for 30 min. After electrophoresis, the slides were placed in a staining tray and covered with a pH 7.5 Tris-HCl neutralizing buffer in the dark for 5 min. This last step was repeated 3 times. The slides were then drained, overlayered with 20 μg/mL EtBr, covered with coverslips, and examined under a fluorescence microscope at a magnification of 400 times. All the slides were coded and examined blindly. Routinely, 100 cells were examined per animal.

2.5 Statistical analysis The DNA length-to-width ratios were used in all comparisons (Clements *et al*., 1997; Ralph and Petras, 1998; Rajaguru *et al.*, 2001). Data were obtained from duplicate slides. The results from the 5 treated groups were compared with those from negative control groups using non-parametric comparisons (Kruskal-Wallis test). The extent of intercellular heterogeneity within each of the data sets was determined from the range of the DNA length-to-width ratios to the standard deviation (SD) of these ratios. The values below 2 or above 6 indicated the data to be extremely homogeneous or extremely heterogeneous (Vijayalaxmi *et al*., 1992). Linear regression analyses were performed to establish the relationship between the dosages of the two substances and induced DNA damage. All the data were analyzed using statistical software SPSS 12.0. Values are presented as mean ± standard error of mean (SE), and significance level is set at $\alpha = 0.05$.

3. Results

No death or overt signs of morbidity of the tadpoles were observed after the treatment. The mean values (± SE) of DNA length-to-width ratios are summarized in Table 1 and 2. Both the chemical substances increased the DNA damage observed in the tadpoles in a dose-responsive manner. There were strong linear correlations between the mean DNA length-to-width ratios and the concentrations of the two test substances: Dichlorvos, $y = 0.966 + 0.098\ x$, $r^2 = 0.964$, $P < 0.01$; and butachlor, $y = 0.775 + 0.842\ x$, $r^2 = 0.814$, $P < 0.05$.

The tadpoles exposed to the lower concentrations of butachlor (0.292 mg/L and 0.584 mg/L) did not show a significant increase in the mean DNA length-to-width ratios compared to those of the negative control ($P > 0.05$). However, the tadpoles exposed to the lower concentrations of dichlorvos (2.256 mg/L) showed a significant increase in DNA damage ($P < 0.05$), and the tadpoles exposed to higher concentrations of the two test substances (4.512, 6.768, 9.024 and 11.280 mg/L for dichlorvos, and 0.876, 1.168 and 1.460 mg/L for butachlor) showed a very significant increase in DNA damage ($P < 0.01$). Similarly, the tadpoles exposed to MMS showed a significant increase in DNA damage ($P < 0.01$).

Figure 1 shows the distribution of DNA damage in individual cells. Of the tadpoles treated with increasing concentrations of the two test substances, higher proportions of cells had greater amount of DNA damage than those of the negative control. This visual observation was confirmed by the higher dispersion of the data. In all cases, the range-to-SD ratios (Tables 1, 2) indicated that the intercellular distribution of DNA damage was neither extremely homogeneous, nor extremely heterogeneous. The values ranged between 3.739 and 5.069 for dichlorvos, with an average of 4.320, while those for butachlor between 3.408 and 5.212, with an average of 4.275. The ratios for the negative and positive controls were 4.129 and 4.636, respectively.

Table 2 Detection of DNA damage, measured by the mean DNA length-to-width ratios, in erythrocytes of *R. zhenhaiensis* tadpoles after a 24-h whole-body exposure to dechlorinated water, MMS and various concentrations of butachlor.

	Dose (mg/L)	Number of tested tadpoles	DNA length: width ratio ± *SE*[a]	P[b]	Range: SD ratio
Butachlor	1.460	6	2.268 ± 0.547	<0.001	4.950
	1.168	6	1.726 ± 0.632	<0.001	3.938
	0.876	6	1.265 ± 0.513	<0.01	3.408
	0.584	6	1.043 ± 0.361	>0.05	5.212
	0.292	6	1.016 ± 0.446	>0.05	3.865
Negative control		6	1.018 ± 0.348		4.129
Positive control (MMS 3.125 mg/L)		6	1.898 ± 0.694	<0.001	4.636

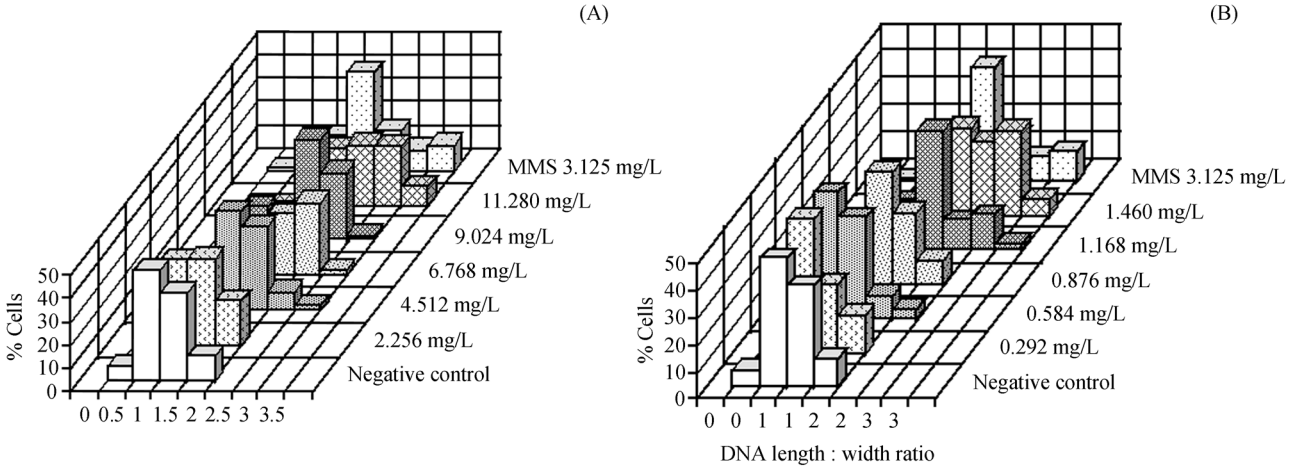

Figure 1 Distribution of DNA damage (based on tail lengths of DNA patterns pooled across 6 tadpoles in each dose group) observed at the cellular level in *R. zhenhaiensis* tadpoles after exposure for a 24-h period to dechlorinated water, MMS and selected concentrations of dichlorvos (A) and butachlor (B)

4. Discussion

Comet assay is currently one of the most commonly used tools to measure genotoxicity of pollutants on organism, and is now widely applied in toxicology, biology, medicine and biomonitoring. It detects DNA damage in individual cells (Olive *et al*., 1990; Clements *et al*., 1997). Since relatively few cells are required (i.e., a few hundreds), any of the cells that have a nucleus can be used, and the assay has been shown to be sensitive to a large number of mutagens (Rajaguru *et al*., 2001). In addition, the ability of this procedure to identify "sensitive" cells in an otherwise normal population of cells permits analysis of low dose-responsive relationships (Tice, 1995). Our results prove these properties again. In the present study, commercial preparations of the agents were tested because of these forms to which organisms are exposed in their natural environment.

The toxicity of dichlorvos has been reported in numerous studies (Cakira and Sarikaya, 2005; Geng *et al*., 2005b; Booth *et al*., 2007). A study that was conducted with appropriate experimental design, although limited by its poor survival rate, indicated a significant increase in incidence of stomach tumors in female mice and pancreatic tumors in male rats that were fed with dichlorvos (Chan *et al.*, 1991). Wooder and Wright (1981) reported that dichlorvos was an alkylating agent, and as such it can potentially induce mutations. Dichlorvos was found to induce chromosome aberrations and increase sister chromatid exchanges in human lymphocytes (Shen *et al*., 1989). According to these results and our findings, including our previous study (Geng *et al*., 2005a, 2006), this pesticide has a negative impact on the amphibian populations and environment.

Although numerous studies have reported the toxicity of butachlor, little information is available on its genotoxicity (Geng *et al*., 2005a). It has been shown that bioaccumulation of a herbicide can occur in fish, clam, and shrimp (Wang *et al*., 1992). Butachlor can induce erythrocyte alterations in the catfish *Clarias batrachus* and abnormality of human lymphocyte chromosomes (Ateeq *et al*., 2002). Our findings are consistent with these results and indicate that butachlor causes DNA damage in tadpoles.

Many studies show that amphibian tadpoles are susceptible to genetic damage caused by short-term exposure to low concentrations of chemicals (Ralph *et al*., 1996; Clements *et al*., 1997). Because of preferring small water bodies (e.g., creeks, ponds and drainage ditches) as their breeding habitats, similar exposure to and impact on these tadpoles can occur in the natural environment. Combined with the field studies (Ralph *et al*., 1996), our study shows amphibian tadpoles may be considered as a sensitive biomonitor for detecting the genotoxic potential of pesticide and herbicide contaminants.

In conclusion, dichlorvos and butaclor, because of their genotoxicity at relatively low concentrations, dose-dependent responses, frequent application and broad array of uses, likely pose a threat to organisms inhabiting in small water bodies receiving the runoff with pesticides.

Acknowledgements We thank Dr. Huang X H for her

helping to improve the English of this article. The research was granted by the Natural Science Foundation of Fujian, China (2009J01129) and the Education Department Foundation of Fujian, China (2007F5033).

References

Ankley G. T., Tietge J. E., Defoe D. L., Jensen K. M., Holcombe G. W., Durhan E. J., Diamond S. A. 1998. Effects of ultraviolet light and methoprene on survival and development of *Rana pipiens*. Environ Toxicol Chem, 17: 2530–2542

Ateeq B., Abul F. M., Niamat A. M., Ahmad W. 2002. Induction of micronuclei and erythrocyte altertions in the catfish *Clarias batrachus* by 2, 4 - dichlorphenoxyacetic acid and butachlor. Mutat Res-Gen Toxicol Environ Mutagene, 518: 135–144

ATSDR. 1997. Toxicological profile for dichlorvos (Atlanta, GA: Agency for toxic substances and disease registry), NTIS PB98-101124, 201 pp

Beebee T. J. C., Griffiths R. A. 2005. The amphibian decline crisis: A watershed for conservation biology? Biol Conserv, 125: 271–285

Berrill M., Bertram S., Pauli B. 1997. Effects of pesticides on amphibian embryos and tadpoles. In: Green D. M. (ed). Amphibians in decline: Canadian studies of a global problem. Society for the Study of Amphibians and Reptiles, St. Louis, MO. 233–245

Booth E. D., Jonesa E., Elliotta B. M. 2007. Review of the *in vitro* and *in vivo* genotoxicity of dichlorvos. Regul Toxicol Pharmacol, 49: 316–326

Cai M. Z. 1979. Observations on reproductive habits of thirty-two anuran species of Fujian Province. J Fujian Normal Univ, 1: 71–79 (In Chinese with English abstract)

Cakira S., Sarikaya R. 2005. Genotoxicity testing of some organophosphate insecticides in the *Drosophila* wing spot test. Food Chem Toxicol, 43: 443–450

Chan P. C., Huff J., Haseman J. K., Alison R., Prejean J. D. 1991. Carcinogenesis studies of dichlorvos in Fischer rats and B6C3F1 mice. Jap J Cancer Res, 82: 157–164

Clements C., Ralph S., Petras M. 1997. Genotoxicity of select herbicides in *Rana catesbeiana* tadpoles using the alkaline sing-cell gel DNA electrophoresis (Comet) assay. Environ Mol Mutagen, 29: 277–288

Davidson C. 2004. Declining downwind: Amphibian population declines in California and historical pesticide use. Ecol Appl, 14: 1892–1902

Duellman W. E., Trueb L. 1994. Biology of amphibians. Baltimore: The John Hopkings University Press, 1–610

Geng B. R., Yao D., Huang H., Xue Q. Q., Lian Y., Zheng Z. 2005. Acute toxicities and effects of dichlorvos and butachlor to *Rana japonica* tadpoles and its growth. Herpetol Sinica, 10: 127–132. (In Chinese with English abstract)

Geng B. R., Yao D., Xue Q. Q. 2005a. Genotoxicity of the pesticide dichlorvos and herbicide butachlor in *Rhacophoridae megacephalus* tadpoles. Acta Zool Sinica, 51: 447–454

Geng B. R., Yao D., Xue Q. Q. 2005b. Acute toxicity of the pesticide dichlorvos and the herbicide butachlor to tadpoles of four anuran species. Bull Environ Contamin Toxicol, 75: 343–349

Geng B. R., Xue Q. Q., Lian Y., Yao D. 2006. Comparison of sensitivity to Dichlorvos of *Rhacophorus megacephalus* tadpole at different developmental stages. Acta Zool Sinica, 52: 892–898

Gosner K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica, 16: 183–190

Hu X. X. 1998. The current situation and developing trends of pesticide industry in China. Pesticides, 37: 7–10 (In Chinese with English abstract)

Mann R. M., Bidwell J. R. 2001. The acute toxicity of agricultural surfactants to the tadpoles of four Australian and two exotic frogs. Environ Poll, 1l4: 195–205

Olive P. L., Banath J. P., Durand R. E. 1990. Heterogeneity in radiation-induced DNA damage and repair in tumor and normal cells using the comet assay. Rad Res, 122: 86–94

Rajaguru P., Kalpana R., Hema A., Suba S., Baskarasethupathi B., Kumar P. A., Kalasielvi K. 2001. Genotoxicity of some sulfur dyes on tadpoles (*Rana hexadactyla*) measured using the comet assay. Environ Mol Mutagen, 38: 316–322

Ralph S., Petras M., Pandrangi R.,Vrzoc M. 1996. Alkaline single cell gel (comet) assay and genotoxicity monitoring using two species of tadpoles. Environ Mol Mutagen, 28: 112–120

Ralph S., Petras M. 1998. Comparison of sensitivity to methyl methanesulphonate among tadpole developmental stages using the alkaline single-cell gel electrophoresis (comet) assay. Environ Mol Mutagen, 31: 374–382

Shen Z. C., Li Y. C., Huang Z. J., Jiang H. Z., Chen C. S., Zhang P. 1989. The studies on the chromosmoe aberrance and sister-chromatid exchange in DDVP produce workers. Acad J Sec Mil Med Univ, 10: 36–39 (In Chinese with English abstract)

Stuart S. N., Chanson J. S., Cox N. A., Young B. E., Rodrigues A. S., Fischman D. L., Waller R. W. 2004. Status and trends of amphibian declines and extinctions worldwide. Science, 306: 1783–1786

Tice R. R. 1995. Applications of the single cell gel assay to environmental biomonitoring for genotoxic pollutants. In: Butterworth B. M., Corkum L. D., Guzma'n-Rinco'n J. (eds). Biomonitoring and bio-markers as indicators of environmental change. New York: Plenum Press, 69–79

Vijayalaxmi, Tice R. R., Strauss G. H. 1992. Assessment of radiation-induced DNA damage in human blood lymphocytes using the single-cell gel electrophoresis technique. Mutat Res, 271: 243–252

Wang M. Z., Jia X. Y. 2009. Low levels of lead exposure indu122e oxidative damage and DNA damage in the testes of the frog *Rana nigromaculata*. Ecotoxicology, 18: 94–99

Wang Y. S., Jaw G. C., Tang H. C., Lin T. S., Chen Y. L. 1992. Accumulation and release of herbicide butachlor, thiobencarb, and chlomethoxyfen by fish, clam, and shrimp. Bull Environ Contamin Toxicol, 48: 474–480

WHO. 1989. Environmental health criteria 79: Dichlorvos. In Dichlorvos. Geneva: World Health Organization, 1–157

Wooder M. F., Wright A. S. 1981. Alkylation of DNA by organophosphorus pesticides. Acta Pharmacol Toxicol, 49: 51–55

Xie F., Lau M. W. N., Stuart S. N., Chanson J. S., Cox N. A., Fischman D. L. 2007. Conservation needs of amphibians in China: A review. Science in China Series C: Life Sci, 50: 265–276

Yao B., Xu J. M., Zhang C. L. 2003. Behavior of herbicide butachlor in environment. Ecol Environ, 12: 66–70 (In Chinese with English abstract)